NASA Technical Memorandum 103853

In Vitro Measurement of Nucleus Pulposus Swelling Pressure: A New Technique for Studies of Spinal Adaptation to Gravity

A. R. Hargens, Ames Research Center, Moffett Field, California
M. G. Glover, M. M. Mahmood, S. Gott, and S. R. Garfin, University of California, San Diego, California
R. Ballard and G. Murthy, Ames Research Center, Moffett Field, California
M. D. Brown, University of Miami, Miami, Florida

January 1992

National Aeronautics and Space Administration

Ames Research Center
Moffett Field, California 94035-1000

In Vitro Measurement of Nucleus Pulposus Swelling Pressure: A New Technique for Studies of Spinal Adaptation to Gravity

A. R. HARGENS, M. G. GLOVER,* M. M. MAHMOOD,* S. GOTT,* S. R. GARFIN,* R. BALLARD, G. MURTHY, AND M. D. BROWN†

Ames Research Center

Summary

Swelling of the intervertebral disc nucleus pulposus is altered by posture and gravity. We have designed and tested a new osmometer for in vitro determination of nucleus pulposus swelling pressure. The functional principle of the osmometer involves compressing a sample of nucleus pulposus with nitrogen gas until saline pressure gradients across a 0.45 micron Millipore filter are eliminated. Swelling pressures of both pooled dog and pooled pig lumbar disc nucleus pulposus were measured on the new osmometer and compared to swelling pressures determined using the equilibrium dialysis technique. The osmometer measured swelling pressures comparable to those obtained by the dialysis technique. This osmometer provides a rapid, direct, and accurate measurement of swelling pressure of the nucleus pulposus.

Introduction

The mechanism of disc degeneration and its role in the etiology of lower back pain is unclear. However, there is much indirect evidence implicating the discs of the lumbar spine in a significant percentage of lower back pain. The swelling pressure of the disc nucleus pulposus may be an important factor in disc degeneration and/or disc herniation. This swelling pressure is caused by the high negative charge of the proteoglycans, creating a negative fluid pressure across the anulus or end plate such that fluid is taken up until the swelling pressure is counterbalanced by the external load on the disc. This pressure maintains disc height and is proportional to the fixed charge density and proteoglycan concentration within the nucleus pulposus. During disc herniation, fluid may be taken up by this tissue and which causes compression of neurovascular structures outside of the disc. Current data on swelling pressures have been obtained by using the technique of equilibrium dialysis, where the swelling pressure is interpolated within the osmotic pressures of polyethylene glycol solutions of different concentrations. This method requires several (3 to 5) tissue slices of 20 to 100 mg each for a single measurement, and 24 to 48 hours for them to equilibrate with the polyethylene glycol solutions into which they are placed. In this paper a new osmometer is described that allows determinations using a reduced amount of nucleus pulposus tissue (a single 5 to 10 mg sample) and a 10- to 30-minute equilibration time. The accuracy and precision of the osmometer measurements were evaluated using the equilibrium dialysis technique as the standard for comparison.

Materials and Methods

Osmometer Technique

The osmometer consists of an acrylic chamber which houses a semipermeable Millipore 0.45-micron, pore size membrane with a watertight seal (fig. 1). The membrane is secured against a fixed volume chamber filled with normal saline that adjoins the diaphragm of a Hewlett-Packard 1280C low volume displacement pressure transducer calibrated to ±100 mm Hg. Five to 10 mg specimens of nucleus pulposus are placed in the sample well at room temperature on the side of the membrane opposite the saline column. A threaded cap to the sample well chamber has connections to a nitrogen source for compressing the disc sample. Pressure is recorded to the nearest 0.1 psi on a Heise precision pressure gauge (50 psi maximum) connected in series to the nitrogen source and osmometer. Transmembrane pressure gradients are continuously measured by a strip-chart recorder.

The curve in figure 2 illustrates the mechanics of measuring swelling pressure using the osmometer. After calibration, the initial pressure on the saline column, as recorded by the pressure transducer, is zero because no pressure gradient exists across the membrane. First a syringe attached to a flexible tube and then tissue paper are used to remove all the saline from the surface of the Millipore membrane and the sample well. This results in a plateau of negative pressure between −10 and −20 mm Hg. The nucleus pulposus is placed in the sample well on top of the membrane opposite the saline column. The sample begins to swell as it imbibes saline from

*University of California, San Diego, California.
†University of Miami, Miami, Florida.

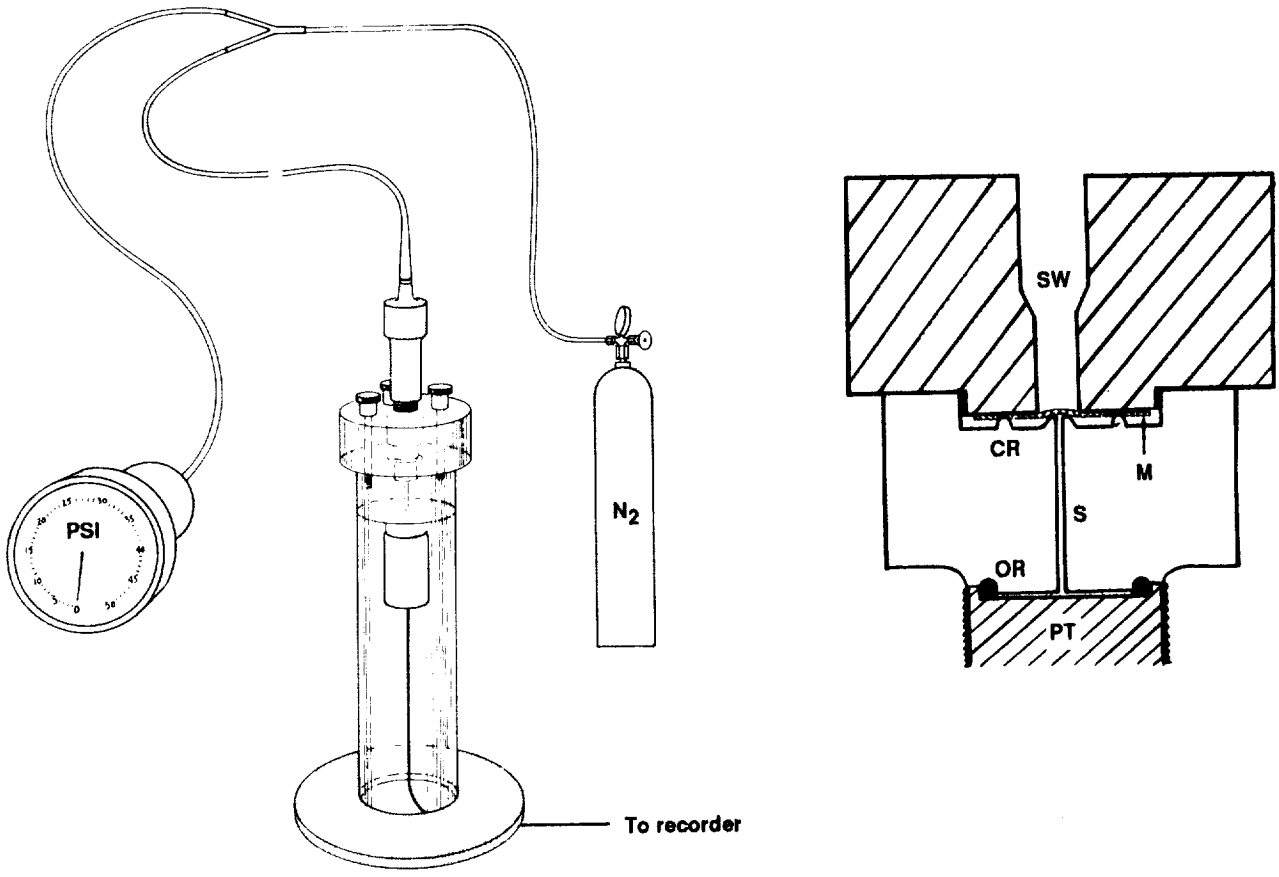

Figure 1. New compression-type osmometer for measuring swelling pressure of nucleus pulposus. Left: Plexiglas osmometer mounted on stand with nitrogen gas inlet at top. Right: Cross-section of osmometer with sealing of membrane (M) by crimp rings (CR) on the screw-down Plexiglas plate. Nucleus pulposus is placed in the sample well (SW) on top of the membrane. Pressure gradients across the membrane are transmitted by the saline fluid column (S) and monitored by the pressure transducer (PT) fitted tightly to the bottom of the osmometer by an O-ring (OR).

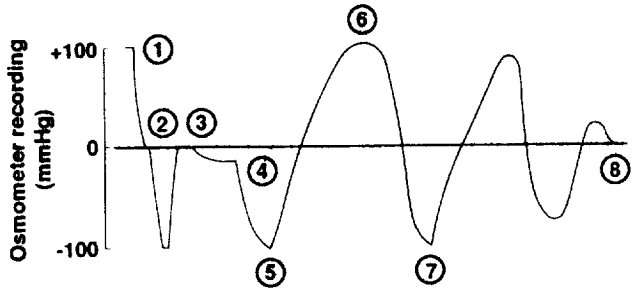

Figure 2. Procedure for measuring swelling pressure of nucleus pulposus.
1) The osmometer is calibrated for +100, 0, and −100 mm Hg.
2) The zero point is checked again with a few microliters of saline atop the membrane.
3) Syringe attached to flexible tube and Kimwipes are used to remove all saline, which results in a plateau between −10 to −20 mm Hg.
4) Nucleus pulposus sample is placed on the membrane.
5) The pressure is allowed to go to the bottom of the scale and nitrogen pressure is applied. The zero is overshot and the nucleus pulposus sample adjusts to the compression.
6) If equilibrium is not reached, a negative pressure reading results and the pressure is allowed again to go to the bottom of the scale.
7) The above steps 5) and 6) are repeated until equilibrium is established.
8) Equilibrium is defined as a zero pressure-transducer recording for at least 60 seconds.

beneath the membrane, which causes pressure below the membrane to fall precipitously. The pressure is allowed to fall to the bottom of the scale (below –100 mm Hg). To counteract the negative pressure in the saline column, nitrogen pressure is applied to the sample of nucleus pulposus until zero on the transducer is "overshot" and the nucleus pulposus' matrix is, therefore, compressed. During this time the pressure transducer senses total tissue pressure (combination of pressures from fluid and solid components of the sample). After a short period, the pressure measured by the transducer begins to fall again. Nitrogen pressure is increased and these steps are repeated until the pressure stabilizes at zero for 60 seconds. This "stable" reading is recorded as the equilibrium swelling pressure. At this point, the applied nitrogen pressure balances the swelling pressure of the nucleus pulposus and nullifies the pressure gradients across the Millipore filter.

Study Method

Lumbar spines were obtained from a freshly-killed 35-kg mongrel dog and two 35-kg mini-pigs and frozen at –70°C. When needed, the frozen spine was cut transversely through each vertebral body on a band saw. The posterior elements were also removed. The intact discs and end-plates were then partially thawed in a 100% humidity environment. The spine segments were incised and nucleus material was extracted while still partially frozen (fig. 3). For the dog, five levels were pooled and mixed thoroughly. Six levels were collected and pooled for the pig. Following mixing, wet and dry weight determinations were made for each species by weighing a wet sample and then drying to constant weight in a 125°C drying chamber. The mixed nucleus pulposus of each species was divided into samples for osmometer and equilibrium dialysis determinations of swelling pressure. Five separate samples were tested and compared. Additionally, samples of the pooled pig nucleus were measured repeatedly ten times to test the reproducibility of the osmometer.

Comparison of Osmometer to Equilibrium Dialysis Technique

Sections of cut 2000-MW (molecular weight) pore size dialysis tubing were prepared by tying off one end with 2-0 nylon monofilament suture and were then weighed. A small sample of nucleus pulposus was then placed into the tube and the tube was weighed again (the sample approximated 47% of the total weight). The change in weight represented the weight of the enclosed nucleus pulposus. The open end was then tied with 2-0 nylon suture after evacuation of air, and the composite weighed after trimming the ends (fig. 4). A size matched control was also prepared, differing only in that it contained no nucleus pulposus. Both were placed in molal solutions of 8000 MW polyethylene glycol (PEG) at the following concentrations: 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, 0.6, and 0.8 g PEG/ml water. The manufacturer of our 8000 MW PEG (Sigma, Inc.) indicated that their previously-prepared 6000 MW PEG was actually 8000 MW based upon recent, better techniques for measuring MW. Hence, we used the same virial coefficients for our PEG as those provided by Edmond and Ogston (1968). The PEG solutions containing the dialysis tubes were placed in the

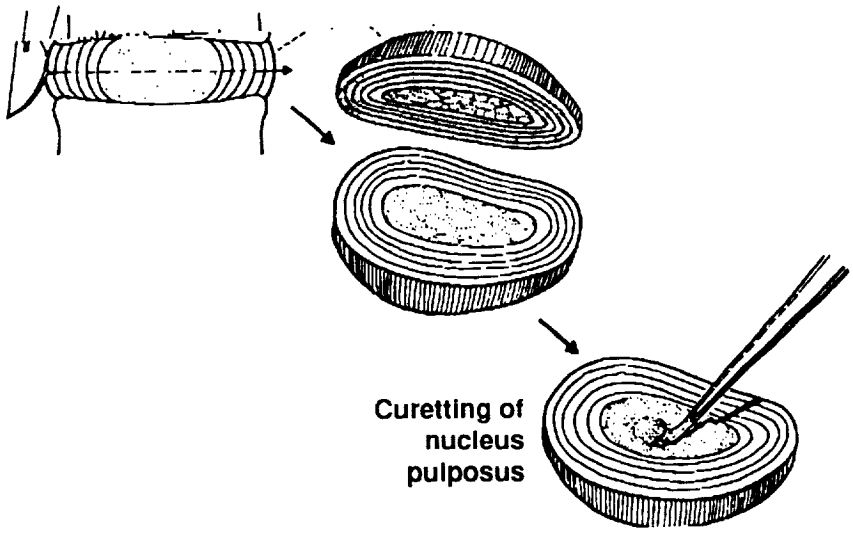

Figure 3. The intervertebral disc plus the adjacent end-plates are isolated on a band saw and the frozen anulus incised. The frozen nucleus pulposus is removed with a curette and returned to the freezer at –70° Celsius.

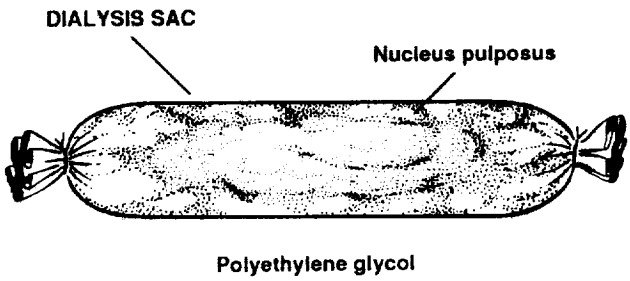

Figure 4. The sample of nucleus pulposus is placed in the 2000-MW cutoff dialysis tubing and all excess air is expelled. Weights are determined after each step of preparation. The composite is placed in a solution of polyethylene glycol for 48 hours.

refrigerator at 4°C (277°K) for 48 hours. One hour after removal from refrigeration, each tube was carefully blotted dry and weighed until five consecutive weighings differed by less than 0.005 grams. The mean of the five weights was calculated and used for further comparisons. Calculations of the equilibrium swelling pressure consisted of determining the pre- and post-PEG solution exposure weights of the nucleus pulposus minus water content due only to the dialysis tube itself (determined from the size matched control). From this the percent weight change in the nucleus was calculated for each PEG concentration and the results plotted (fig. 5). A logarithmic curve fitting procedure was employed and the X-intercepts were calculated for the pig and dog. These values were used in the virial expansion equations for macromolecular solutions to determine the osmotic pressures of the PEG solution that produced no net weight change in the nucleus pulposus. This osmotic pressure was taken as equal to the swelling pressure of the nucleus pulposus. The swelling pressure values obtained with equilibrium dialysis were compared to the mean values obtained with the new osmometer.

Results

Mean pressures of 2.3 ± 0.3 atm and 0.5 ± 0.02 atm were obtained for the dog and pig nucleus pulposus, respectively, as measured with the osmometer (table 1 and fig. 6). This agreed well with the equilibrium dialysis technique which gave pressures of 2.03 atm and 0.49 atm for the dog and pig pooled nucleus pulposus specimens, respectively (both corrected to a room temperature of 296°K). In figure 6, values are presented as means ±S.E. for osmometer results and calculated values for the equilibrium dialysis determination. The dog disc material had a lower water content than that of the pig (81% vs. 90%, respectively).

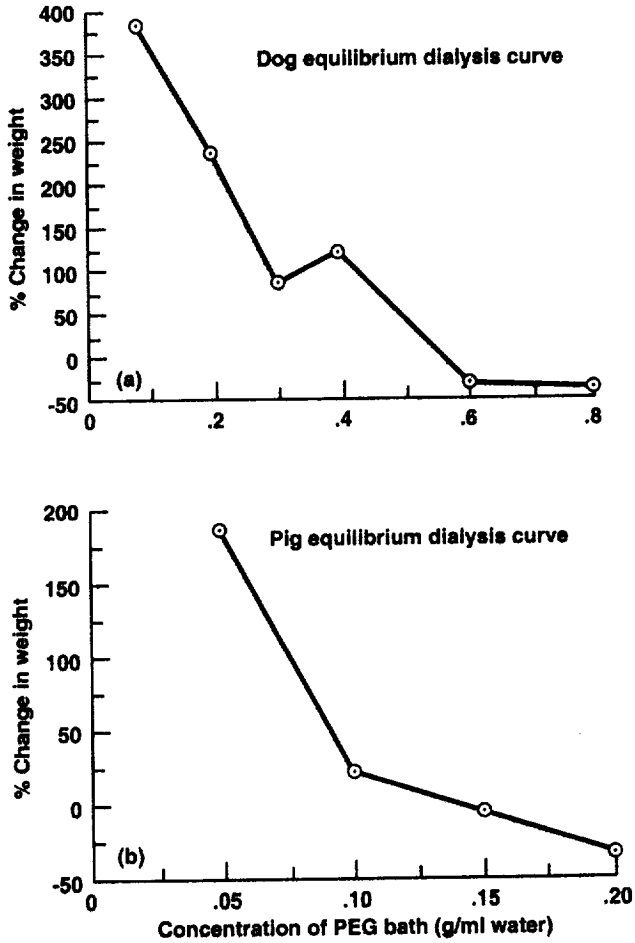

Figure 5. The percent change in weight of the dog (a) and pig (b) nucleus pulposus when allowed to swell in the dialysis sac immersed in PEG is plotted against the concentration of the PEG bath.

The ten samples, measured repeatedly in the osmometer, yielded a mean swelling pressure of 0.53 ± 0.01 atm with a variance of 0.0005 for pig nucleus (table 2). This demonstrates the high reproducibility of the osmometer. An individual swelling pressure measurement in the osmometer required 10 to 30 minutes which depended upon the magnitude of swelling pressure for a given sample.

Discussion

Proteoglycans are the major nonaqueous components of the nucleus pulposus of the intervertebral disc. They are complex structures with variable molecular weights up to 10^8 daltons (average 6×10^5) (Bushell et al., 1977) whose components include chondroitin sulphate, keratin sulphate, hyaluronic acid, core protein, and link protein

Table 1. Swelling pressures of dog and pig nucleus pulposus using osmometer

Dog disc	
Trial No.	Swelling pressure (atm)
1	2.1
2	2.8
3	2.9
4	1.2
5	2.4
Mean (± S.E.)	2.3 ± 0.3
Water Content = 81%	
Pig Disc	
Trial No.	Swelling pressure (atm)
1	0.5
2	0.5
3	0.4
4	0.5
5	0.5
Mean (± S.E.)	0.5 (± 0.02)
Water Content = 90%	

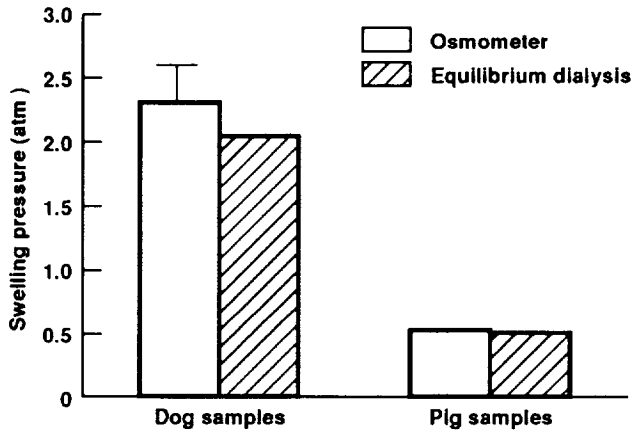

Figure 6. Results of the osmometer technique and equilibrium dialysis technique for measuring the swelling pressures of the dog and pig nucleus pulposus.

(Muir, 1979). The negative charges associated with chondroitin sulphate and keratin sulphate are largely responsible for the swelling pressure generated by the nucleus pulposus. These negative charges maintain hydration of the nucleus pulposus in the face of high external loads. Also, proteoglycan macromolecules are closely packed, and thus impart a low hydraulic permeability and retard fluid loss from the nucleus pulposus (Maroudas and Urban, 1980).

Measurement of swelling pressure (P_S) has been a difficult task because of practical and conceptual problems (Maroudas and Urban, 1980) and many methods have been employed (Charnley, 1952; Nachemson, Elfstrom, 1970; Tanford, 1961). Much work in this area has been performed by Maroudas and Urban, who found that equilibrium hydration studies correlate best with the effective osmotic pressure of nucleus pulposus material (Maroudas and Urban, 1980). Equilibrium dialysis, as a method for determining swelling pressures, is based on the concept of calculating osmotic pressure of simple macromolecular solutions and then using these solutions as a standard against which water loss or gain of nucleus pulposus samples contained within dialysis tubing can be compared. Determination of the concentration of a reference solution that will cause no net gain or loss of water allows one to interpolate the swelling pressure of the

Table 2. Reproducibility of osmometer using pig nucleus pulposus

Trial No.	Swelling pressure (atm)
1	0.59
2	0.54
3	0.51
4	0.52
5	0.53
6	0.53
7	0.53
8	0.53
9	0.52
10	0.53
Mean ± S.E.	0.53 ± 0.01 atm
Variance	0.00048

material enclosed in the dialysis tube. Calculation of the reference solution pressures is based on the virial expansion for osmotic pressure (π):

$$\pi = RTc/MW(1 + Ac/MW + ...)$$

where

R	Universal gas constant
T	Absolute temperature (°K)
c	Grams of macromolecule/gram solvent
MW	Molecular weight of macromolecule
A	Second virial coefficient (moles^2)

The second virial coefficient consists of three independent terms: the Donnan term, the excluded volume term, and the interaction between the charged macromolecule and electrolyte plus a self-attraction term of the electrolyte itself. The Donnan term is a measure of the imbalances in the concentrations of diffusible electrolytes resulting from the fixed charge groups on the macromolecule. In other words, the polyelectrolytes on one side of the membrane co-exist with a relatively lower concentration of small ions of like charge and a relatively higher concentration of small ions of opposite charge (Tanford, 1961).

We used equilibrium dialysis swelling pressures as a standard against which to compare our results. We also employed polyethylene glycol (PEG) as a reference macromolecule (Maroudas and Urban, 1980). A PEG of 8000 MW was used rather than Maroudas and Urban's 20,000 MW because we were able to obtain a more uniform average MW with the 8000 MW PEG. The equilibrium dialysis technique is useful, reliable, and accurate. However, this technique has practical limitations such as time required for a single determination, its indirect approach, and relatively large amount and number of samples needed. Because we used the virial coefficients derived by Edmond and Ogston (1968), we were obliged to dilute our PEG solutions in a manner identical to theirs (i.e., by dilution with water) in order for us to employ their virial coefficients correctly. Maroudas and Urban diluted their PEG solutions with 1.5 and 0.15 M NaCl and found relatively large differences in osmotic pressure between these two media (Maroudas and Urban, 1980). However, at ionic strengths lower than 0.15 M, differences between the two media would be expected to be less. Despite the differences between the external media in contact with the nucleus pulposus samples across the membrane, we obtained similar results for the direct osmometric and indirect equilibrium dialysis techniques. Whenever possible, it is better to measure a colligative property of a solution or gel directly; this can be done with our new osmometric technique. Despite the differences in the direct (osmometer) and indirect (equilibrium dialysis) techniques in terms of bathing media, membrane pore size, and errors inherent in weighing small tubes and using empirically-derived virial coefficients, we find the agreement between the two techniques quite remarkable.

Our purpose was to find a method for measuring the swelling pressure of the nucleus pulposus that gave rapid results, used small samples, and provided a directly measured rather than calculated or interpolated pressure. As demonstrated, we have accomplished this with an osmometer, modified so that nitrogen gas is used to compress and directly cancel the swelling pressure of the nucleus pulposus sample. The nitrogen pressure overcomes problems associated with high negative pressures in the saline column beneath the membrane, namely cavitation due to the large swelling pressure of the nucleus pulposus. This also obviates the problem of measuring high negative pressures with standard, readily-available pressure transducers that are usually nonlinear and

inaccurate below −100 mm Hg. When the applied nitrogen pressure achieves equilibrium across the membrane, there is negligible saline movement between the nucleus pulposus sample and the opposing saline column. However, in approaching equilibrium, it is possible that our samples were diluted somewhat by saline transport from the pressure-transducer side of the membrane. This would, in effect, lower the swelling pressure of the sample. However, we were very careful to dry the membrane well before applying the sample. We have minimized drying and loss of matrix constituents by the sample of nucleus pulposus by (l) minimizing the time to reach equilibrium (10 to 30 minutes), (2) using a deep and narrow sample well inside a closed chamber, and (3) checking wet and dry weights of homogenized samples (some placed on the osmometer and others not) and finding that the dry/wet ratio is not affected by the measurement. Furthermore, whereas conventional osmometers allow saline transport into the sample to set up a negative pressure on the transducer side of the membrane, our osmometer minimized such saline transport by compression of the sample at a level equal to its swelling pressure. Nucleus pulposus swelling pressure measurements obtained using the osmometer correlated well with the osmotic pressure values determined by equilibrium dialysis of the corresponding samples. The results for dog and pig nucleus in this study show good correlation and provide evidence of the accuracy and reproducibility of the osmometer in measuring swelling pressures. The absolute values of swelling pressure for these animals are relatively low, probably because the dogs and pig were anesthetized for several hours prior to tissue collection. The precision is demonstrated by the low coefficient of variation (0.041) for a series of measurements on portions of a pooled sample.

Conclusions

The new osmometer accurately and directly measures the swelling pressure of nucleus pulposus. The pressurized osmometer provides results that correlate well with the equilibrium dialysis technique. Dog nucleus pulposus pressures were 2.3 atm versus 2.0 atm and the pig nucleus pulposus was 0.5 atm versus 0.5 atm, respectively, for osmometer and dialysis techniques. The osmometer technique is reproducible (variance of 0.0005) and is much faster than the equilibrium dialysis technique, requiring one-half hour or less per observation compared to 48 hours for equilibrium dialysis. Osmometry allows direct pressure measurements with a single 5 to 10 mg sample as opposed to a calculated pressure determination requiring multiple 20 to 100 mg samples.

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 1992	Technical Memorandum

4. TITLE AND SUBTITLE

In Vitro Measurement of Nucleus Pulposus Swelling Pressure: A New Technique for Studies of Spinal Adaptation to Gravity

5. FUNDING NUMBERS

199 14 12 04

6. AUTHOR(S)

A. R. Hargens, M. G. Glover,* M. M. Mahmood,* S. Gott,* S. R. Garfin,* R. Ballard, G. Murthy, and M. D. Brown†

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Ames Research Center
Moffett Field, CA 94035-1000

8. PERFORMING ORGANIZATION REPORT NUMBER

A-91108

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

National Aeronautics and Space Administration
Washington, DC 20546-0001

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

NASA TM-103853

11. SUPPLEMENTARY NOTES

Point of Contact: Alan Hargens, Ames Research Center, MS 239-11, Moffett Field, CA 94035-1000
(415) 604-5746 or FTS 464-5746
*University of California, San Diego, California †University of Miami, Miami, Florida

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Unclassified-Unlimited
Subject Category – 51

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 words)***

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14. SUBJECT TERMS	15. NUMBER OF PAGES
Nucleus pulposus, Swelling pressure, Intervertebral disc, Spinal pain	8
	16. PRICE CODE: A02

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified		